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Malate plays a central role in plant nutrition

J. Schulze[1,2], M. Tesfaye[3], R. H. M. G. Litjens[4], B. Bucciarelli[2], G. Trepp[2,5], S. Miller[2], D. Samac[3,6], D. Allan[7] & C. P. Vance[2,6,8]

[1]*Institut fuer Bodenkunde und Pflanzenernaehrung der Martin-Luther-Universitaet, Halle-Wittenberg, Halle, Germany.* [2]*Department of Agronomy and Plant Genetics, University of Minnesota, 1991 Upper Buford Circle, St. Paul, MN 55108, USA.* [3]*Department of Plant Pathology, University of Minnesota, 1991 Upper Buford Circle, St. Paul, MN 55108, USA.* [4]*Fontys University, Eindhoven, Eindhoven, The Netherlands.* [5]*ETH Zurich, Switzerland.* [6]*USDA-ARS, Plant Science Research Unit, 1991 Upper Buford Circle, 411 Borlaug Hall, St. Paul, MN 55108, USA.* [7]*Department of Soil, Water, and Climate, University of Minnesota, 1991 Upper Buford Circle, St. Paul, MN 55108, USA.* [8]*Corresponding author**

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Abstract

Malate occupies a central role in plant metabolism. Its importance in plant mineral nutrition is reflected by the role it plays in symbiotic nitrogen fixation, phosphorus acquisition, and aluminum tolerance. In nitrogen-fixing root nodules, malate is the primary substrate for bacteroid respiration, thus fueling nitrogenase. Malate also provides the carbon skeletons for assimilation of fixed nitrogen into amino acids. During phosphorus deficiency, malate is frequently secreted from roots to release unavailable forms of phosphorus. Malate is also involved with plant adaptation to aluminum toxicity. To define the genetic and biochemical regulation of malate formation in plant nutrition we have isolated and characterized genes involved in malate metabolism from nitrogen-fixing root nodules of alfalfa and those involved in organic acid excretion from phosphorus-deficient proteoid roots of white lupin. Moreover, we have overexpressed malate dehydrogenase in alfalfa in attempts to improve nutrient acquisition. This report is an overview of our efforts to understand and modify malate metabolism, particularly in the legumes alfalfa and white lupin.

Introduction

Malate is a key product of plant metabolism and thought by many (see Lance and Rustin, 1984; Martinoia and Rentsch, 1994) to be the ultimate product of glycolysis, rather than pyruvate. Functional roles for malate in plants are quite diverse including, but not limited to: respiration and energy generation, photosynthesis (both C_3 and C_4), fatty acid oxidation, lignin biosynthesis, pulvinal and stomatal function, nitrogen (N_2) fixation and amino acid biosynthesis, ion balance, uptake of phosphorus (P) and iron (Fe), and aluminum (Al) tolerance (Gietl, 1992; Kochian, 1995; Martinoia and Rentsch, 1994).

In N_2-fixing nodules, malate is the predominant source of energy for bacteroid respiration (Driscoll and Finan, 1993) and provides a significant portion of the carbon skeletons for assimilation of fixed N_2 (Rosendahl et al., 1990). Malate may also be involved in regulation of the nodule oxygen diffusion barrier through an osmoelectrical mechanism (Denison, 1998; Galvez et al., 2000; Vance and Heichel, 1991). The critical role that malate plays in root nodules is evidenced by the fact that ineffective nodules, whether induced by changes in either the bacterial or plant genotype, have strikingly reduced levels of malate as compared to effective nodules. Moreover, mutations in rhizobia that block organic acid use res-

* FAX No: +1-651-649-5058
E-mail: vance004@tc.umn.edu

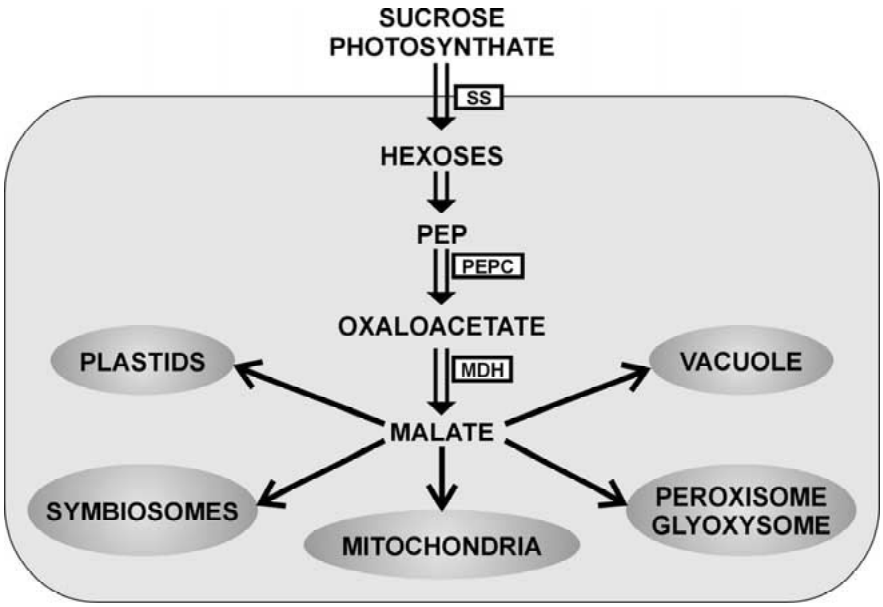

Figure 1. Diagrammatic scheme for the degradation of photosynthate (sucrose) to malate. Due to its diverse functions in plants, malate is targeted to many subcellular locations. Because malate functions in a wide range of processes in distinct cellular locations, at least five forms of malate dehydrogenase (MDH) can be found in plants including: cytosolic, glyoxysomal, peroxisomal, plastidic, and mitochondrial. In root nodules, MDH may also be associated with symbiosomes. SS=sucrose synthase; PEPC=phospho*enol*pyruvate carboxylase.

ult in ineffective nodules while those that block amino acid and carbohydrate use generally have no effect on N_2 fixation (Driscoll and Finan, 1993; Ronson et al., 1981).

Phosphorus is the limiting element in many soils (Bieleski, 1973; Vance, 2001). In such soils plants display adaptive features that aid in acquisition of P (Raghothama, 1999; Schactman et al., 1998). White lupin forms a unique root developmental adaptation as a substitute for a mycorrhizal association, the development of proteoid or cluster roots (Skene, 2001; Watt and Evans, 1999). Proteoid roots not only increase the root surface area by more than 100-fold to aid in exploration for P but they also release phenomenal amounts of organic acids, primarily malate and citrate (Dinkelaker et al., 1995; Skene, 2001). Release of organic acids helps to increase the availability of mineral P by solubilizing Ca-, Fe- and Al-phosphates (Ryan et al., 2001). Exudation of malate and citrate from proteoid roots may represent as much as 25% of total photosynthate (Gardner et al., 1983; Johnson et al., 1996a).

Synthesis of malate is catalyzed by the enzyme malate dehydrogenase (MDH: EC 1.1.1.82) through the reversible reduction of oxaloacetate to malate. Because this enzyme is important in several metabolic pathways in higher plants, it occurs in multiple forms that differ in subcellular localization (Figure 1) and cofactor specificity (Gietl, 1992). Although the reaction favors malate production, whether oxaloacetate or malate forms depends upon physiological conditions and enzyme location.

Chloroplasts contain an NADP-dependent MDH (plMDH) that plays a critical role in balancing reducing equivalents between the cytosol and stroma. Plants also contain at least 4 NAD-dependent MDHs which are found in: (i) the cytosol (cMDH) and peroxisomes (pMDH) involved in malate-aspartate shuttles; (ii) the mitochondria (mMDH) involved in the TCA cycle; (iii) the glyoxysomes (gMDH) functioning in β-oxidation (Gietl, 1992; Miller et al., 1998); and (iv) root nodules (neMDH) functioning in N_2-fixation and N assimilation (Miller et al., 1998). The enzyme has been purified from several plant sources and antibodies produced against plMDH, gMDH, and pMDH. The p and gMDHs are serologically indistinguishable, while the plMDH is antigenically unique.

Because malate plays such a pivotal role in nitrogen-fixing root nodules and in acquisition of P by proteoid roots, we thought it important to understand the biochemical and genetic factors affecting malate synthesis. This report provides an overview of our laboratory's efforts to understand and modify malate synthesis.

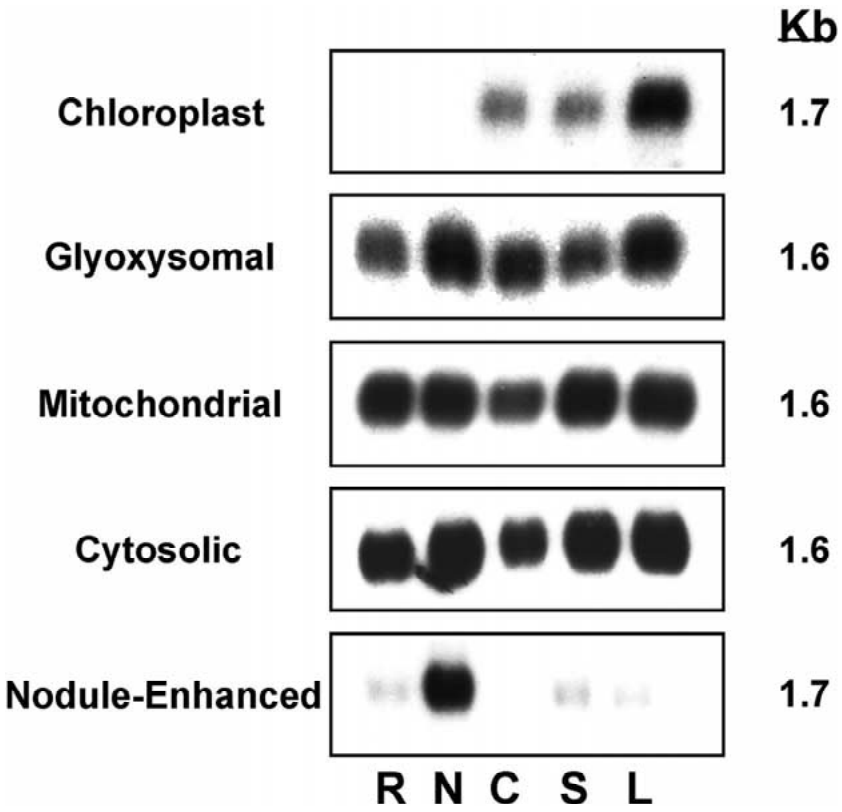

Figure 2. Steady state transcript abundance of the various forms of malate dehydrogenase (MDH) in alfalfa organs: R=root; N=nodule; C=cotyledon; S=stem; L=leaf. Transcript size in kb is noted to the right of each blot. Equivalent amounts of polyA$^+$RNA, 1 μg, were loaded in each lane as measured by ^{32}P poly(U) hybridization (adapted from Miller et al. 1998).

Alfalfa root nodules

Using strategies involving complementation of an *E. coli* MDH$^-$ mutant, RT-PCR, and screening with heterologous probes, we cloned five distinct MDH cDNAs (Miller et al., 1998). These cDNAs encoded chloroplast (pl), mitochondrial (m), glyoxysomal (g), cytosolic (c), and nodule-enhanced (ne) MDHs. The cDNAs representing c- and neMDH were novel, this being the first report of their isolation.

RNA blot analysis was used to assess the relative abundance of each MDH mRNA transcript (Figure 2). There appeared to be fairly uniform abundance of mMDH, gMDH, and cMDH in roots, nodules, cotyledons, stems, and leaves. By comparison, plMDH was found only in green tissues, particularly leaves. Transcripts for neMDH were found predominantly in root nodules with very minor amounts in other tissues. The discovery of a nodule form of MDH is consistent with biochemical studies of pea nodules by Appels and Haaker (1988) and lupin nodules by Ratajczak et al. (1989), suggesting a root nodule form of MDH catalyzing high *in vitro* rates of malate production. Moreover, Fedorova et al. (1999) isolated from pea nodules a neMDH cDNA having striking similarity to the alfalfa homolog.

Because the alfalfa neMDH and cMDH are novel forms, we did extensive biochemical characterization of these proteins. Having both forms of MDH expressed in an *E. coli* MDH$^-$ mutant allowed us to isolate the recombinant enzymes, perform kinetics, and produce antibodies. Kinetic parameters for neMDH were strikingly different than those for cMDH. The Km analysis for oxaloacetate showed that neMDH had a 7-fold greater affinity for this substrate as compared to cMDH. The neMDH enzyme turnover rates for malate oxidation and oxaloacetate reduction were 4-fold and 30-fold greater, respectively, than those for cMDH. Even more surprising, the neMDH specificity constants for oxaloacetate and NADH were 70- to 100-fold greater than those for malate and NAD. These data suggest that neMDH catalysis dramatically favors malate production and neMDH may be a useful target to develop plants with modified malate metabolism.

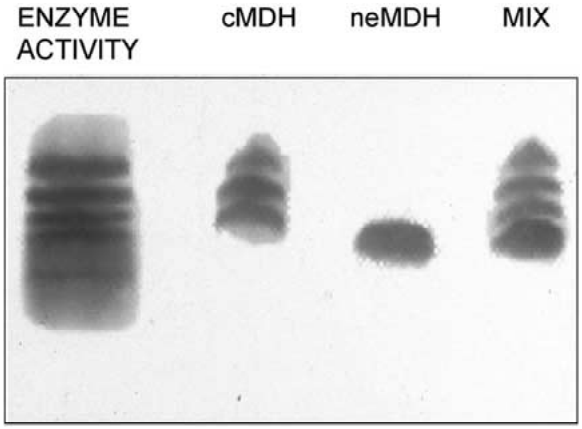

Figure 3. Immunologic specificity of antibodies prepared to cytosolic (c) and nodule-enhanced (ne) malate dehydrogenase (MDH). Nodule protein was subjected to native polyacrylamide gel electrophoresis and then stained for MDH activity (Enzyme Activity) or transferred to nitrocellulose and immunostained with antibodies to cytosolic MDH (cMDH), nodule-enhanced MDH (neMDH), or a mixture of both antibodies (Mix). Note the specificity of c- and neMDH antibodies for specific isozymes of MDH activity.

Antibodies prepared to neMDH and cMDH were highly specific (Figure 3) showing little to no cross reactivity. Having high-quality specific antibodies allowed us to assess steady state quantities of ne- and cMDH protein. Similar to RNA blot analysis, neMDH protein was greater in nodules than any other tissue examined. Substantial amounts of neMDH protein were found in meristematic tissue, with much less in roots, cotyledons, stems, and leaves. Likewise, cMDH protein was uniformly expressed in all tissues examined as were cMDH transcripts. Accompanying immunoprecipitation experiments showed that neMDH comprises the largest proportion of total nodule MDH, about 50%.

Most recently we isolated and sequenced the alfalfa genes encoding *c-* and *neMDH*, respectively (Figure 4). *Cytosolic MDH* was comprised of seven exons interrupted by six introns. This gene structure was conserved in the *Arabidopsis* genome, however, introns in *Arabidopsis* were much smaller than those in alfalfa. Preliminary sequencing of *Medicago truncatula cMDH* showed that intron-exon structure and sequence similarity was very conserved between alfalfa and *M. truncatula*. By comparison, alfalfa *neMDH* contained only one intron, and it was located in the 5′-untranslated region of the gene. Again, intron structure was conserved between alfalfa, *M. truncatula* and *Arabidopsis*.

White lupin proteoid roots

White lupin is adapted for growth on neutral to acidic soils and can flourish in nutrient-poor environments that would limit most plants (Dinkelaker et al., 1995; Gilbert et al., 1998). Although it does not form a mycorrhizal symbiosis, white lupin can effectively acquire soil P through the development of proteoid roots and their subsequent exudation of malic and citric acids (Johnson et al., 1994, 1996a, b; Neumann et al., 2000). Proteoid roots are densely clustered tertiary roots having prolific development of root hairs (Dinkelaker et al., 1995; Skene, 2001). Their formation is suppressed when soil P levels are sufficient, but even when P is adequate, as much as 5–10% of the root mass may be proteoid (Johnson et al., 1984; Skene, 2001). Proteoid root formation can increase the root surface area by more than 100-fold. The malate and citrate exuded from proteoid roots allows for the displacement of bound P from Al^{3+}-, Fe^{3+}- and Ca^{2+}-phosphates, thus increasing available P. A further adaptation to low P is that proteoid roots have enhanced P uptake and increased expression of phosphate transporter genes (Liu et al., 2001; Neumann et al., 1999).

We (Johnson et al., 1994, 1996a, b) as well as others (Keerthisinghe et al., 1998; Neumann et al., 1999) have shown that proteoid roots of white lupin excrete phenomenal amounts of citrate and malate during P-deficiency. The amount of organic acids released in root exudates of white lupin in response to P stress can represent 11–23% of total photosynthate (Dinkelaker et al., 1995; Gardner et al., 1983). We showed, using *in vivo* $^{14}CO_2$-labeling, that during a 70-h period 227 μmol citrate and 156 μmol malate were recovered from −P root exudates as compared to 11 μmol citrate and 9 μmol malate from +P roots (Johnson et al., 1996a). In efforts to understand the mechanisms underlying P-stress induced organic acid exudation from proteoid roots the activity of enzymes related to malate and citrate synthesis was evaluated. We found that increased organic acid synthesis and exudation is mediated by increased specific activities (Table 1) of cMDH, phosphoenolpyruvate carboxylase (PEPC), and citrate synthase (CS) (Johnson et al., 1994, 1996a, b). Increased MDH and PEPC activity in proteoid roots is accompanied by enhanced PEPC and MDH mRNA and protein (Johnson et al., 1996b; C. Uhde-Stone, C. Vance, and D. Allan data not shown). More recently, enhanced citrate synthesis was also shown to be related to reduced degradation as aconitase activ-

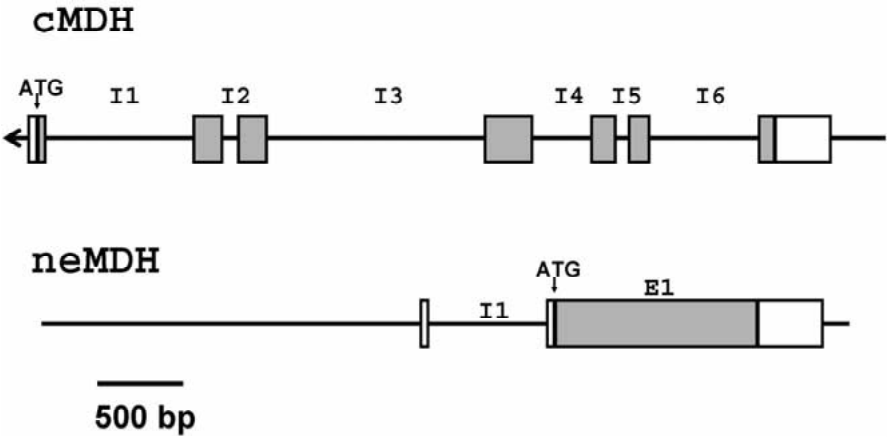

Figure 4. Exon-intron structure of alfalfa cytosolic, *cMDH*, and nodule-enhanced, *neMDH*, genes. The genes are distinctly different in structure, 6 introns are found in the coding region of cMDH while none are found in the coding region of neMDH.

Table 1. Specific activity of malic dehydrogenase (MDH), phosphoenolpyruvate carboxylase (PEPC), and citrate synthase (CS) in proteoid and normal roots of white lupin. Data adapted from Johnson et al. (1994, 1996b) and Gilbert et al. (1998)

Root Type	MDH	PEPC	CS
	$\mu mol\ NADH \cdot min^{-1} \cdot mg\ protein^{-1}$		$\mu mol\ acetyl\ CoA \cdot min^{-1} \cdot mg\ protein^{-1}$
Normal +P	7.9^a	0.08^a	0.11^a
Normal −P	8.2^a	0.27^b	0.08^a
Proteoid +P	7.5^a	0.13^a	0.10^a
Proteoid −P	12.1^b	0.41^c	0.14^b

Each value is the mean of at least nine determinations. Means in each column labeled with the same letter are not different as determined by LSD ($P \leq 0.05$).

Table 2. In vivo CO_2 fixation and recovery of fixed C in root exudates of phosphorus-stressed white lupin. ND=Not Detectable. Adapted from Johnson et al. (1996a)

CO_2 labeling site	Treatment	CO_2 fixation	C recovery		
			Total exudate	Citrate	Malate
		$nmol \cdot min^{-1} \cdot g\ FW^{-1}$	$\mu mol\ C$		
Root	+P	2.6±0.5	0.1±0.1	ND	ND
Root	−P	11.1±1.7	2.4±0.3	1.0±0.6	0.5±0.3
Shoot	+P	72.3±24.7	0.4±0.3	ND	ND
Shoot	−P	102.0±36.1	5.5±1.9	3.5±2.0	1.3±1.3

Mean±SD for five independent determinations. Differences are significant at $P \leq 0.05$.

ity in proteoid roots of P-stressed plants is reduced (Neumann et al., 1999).

In vivo-labeling experiments in which shoots and roots were independently labeled with $^{14}CO_2$ and root exudates subsequently collected (Johnson et al., 1994, 1996a, b) showed that 25–35% of the label found in exuded citrate and malate was derived through root CO_2 fixation via root PEPC (Table 2). For significant amounts of radioactivity to be found in organic acids after root labeling, the oxaloacetate derived from PEPC would require catalysis to malate and citrate via MDH (Figure 1) and CS, respectively. Thus, MDH plays a role in providing organic acids for exudation from P-stressed proteoid roots as an adaptive strategy to acquire unavailable P under infertile conditions.

Transgenic enhancement of malate

Malate has a pivotal role in acquisition of N through symbiotic N_2 fixation and P through root exudation.

Table 3. Steady state mRNA abundance in transgenic plants overexpressing neMDH. Relative abundance measured as incorporation of ^{32}P from labeled probe into neMDH RNA

Transgenic line	Tissue			
	Leaf	Root	Root tip	Nodule
	cpm in neMDH RNA			
Control	1400	1497	2580	7085
MDH 16–27	2360	4169	4537	8649
MDH 20–10	2370	6838	9928	12 600

Mean of two independent determinations.

Table 4. Shoot and root P content of transgenic plants overexpressing neMDH. Data adapted from Tesfaye et al., 2001

Transgenic line	Shoot P	Root P
	$\mu g\ plant^{-1}$	
Control	96^a	34^a
MDH 16–27	136^b	85^b
MDH 20–10	141^b	83^b

Each value is the mean of at least three determinations. Means in each column labeled with the same letter are not different as determined by LSD ($P \leq 0.05$).

Moreover, increased synthesis of citrate and malate is intimately related with plant tolerance to Al stress (Delhaize and Ryan, 1995; Kochian, 1995). Because malate is a critical component of plant nutrient acquisition and adaptation to environmental stress, we hypothesized that improving malate synthesis via transgenic technology might be an effective strategy for improving plant nutrition. Although controversial (Delhaize et al., 2001), precedence for transgenic approaches to increasing organic acid synthesis and improving plant nutrition has been demonstrated by overexpression of citrate synthase (de la Fuente et al., 1997; Koyama et al., 1999; Lopez-Bucio et al., 2000). Overexpression of citrate synthase resulted in plants having improved P nutrition and resistance to Al.

To overexpress MDH we developed a chimeric gene construct with neMDH being under the control of the constitutive 35S promoter. Alfalfa was transformed with the 35S::neMDH construct and transgenic plants recovered. Of nine independent transformants showing increased neMDH activity in root tips, the two showing the greatest enhancement of MDH activity, MDH 20–10 and 16–27, were selected for further study (Tesfaye et al., 2001).

Both lines showed that increased MDH activity was accompanied by greater MDH protein (Tesfaye et al., 2001) and mRNA (Table 3) as compared to the controls. Moreover, during a 24-h period, MDH 16–27 plants exuded significantly more citrate, malate, oxalate, succinate, and acetate into the rhizosphere than did the controls. By comparison, MDH 20–10 plants also tended to exude more organic acids than the control but less than MDH 16–27 (Tesfaye et al., 2001).

To determine whether overexpression of neMDH affected P acquisition and Al tolerance, plants were grown in highly weathered acid soil and hydroponic solution containing various concentrations of Al. The shoot and root P accumulation in transgenic plants was greater than the control (Table 4). Furthermore, root elongation of transgenic plants were significantly more tolerant to Al than was that of the control (Tesfaye et al., 2001).

To assess whether overexpression of neMDH has any effect on N_2 fixation, nodulated plants of MDH 16–27 and a control transgenic line PARC-100 were exposed to $^{15}N_2$ gas for 24 h and N_2 fixation determined. Preliminary results from plants tested at 8 weeks after inoculation showed significantly improved N_2 fixation as evaluated by nodule efficiency (μmol N·gF nodule^{-1}·h^{-1}). The nodule efficiency of PARC-100 was 12.36 while that of MDH 16–27 was 16.33, demonstrating enhanced N_2 fixation potential in nodules of MDH 16–27 plants.

Overview

Malate plays a central role in plant nutrition. Physiological, biochemical, and molecular studies with alfalfa and white lupin provide evidence for the importance of malate in N_2 fixation, acquisition of P from infertile soils, and tolerance to Al stress. At least five separate forms of MDH are found in alfalfa and each is encoded by distinct genes. The unusual kinetic parameters of alfalfa neMDH make it an excellent candidate for enhancing malate synthesis via transgenic technology. Exudation of the organic acids malate and citrate from P-stress induced proteoid roots of white lupin increases the availability of sparingly soluble metal phosphates thereby providing P under low P conditions. Synthesis of malate and citrate in P-stress proteoid roots requires the concerted action of the enzymes PEPC, MDH, and CS. Overexpression of neMDH in alfalfa results in plants with higher P accumulation, improved tolerance to Al, and increased

root nodule efficiency. It will be important to establish whether further enhancement of the expression of neMDH in alfalfa results in improved P uptake and greater tolerance to Al. In addition, it will be critical to determine whether increased nodule efficiency translates into improved N nutrition.

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